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Metastatic clear cell renal cell carcinoma with a long-term response to sunitinib: a distinct phenotype independently associated with low PD-L1 expression

Solène-Florence Kammerer-Jacquet^{1,2}, Angélique Brunot³, Mathilde Lefort⁴, Sahar Bayat⁴, Benoit Peyronnet⁵, Gregory Verhoest⁵, Romain Mathieu⁵, Alexandra Lespagnol⁶, Jean Mosser⁶, Brigitte Laguerre³, Alain Ravaud⁷, Jean-Christophe Bernhard⁸, Frantz Dupuis⁹, Mokrane Yacoub⁹, Marc-Antoine Belaud-Rotureau^{2,10}, Karim Bensalah⁵ et Nathalie Rioux-Leclercq^{1,2}

Short title: Long-term responders in renal carcinoma

Affiliations :

- 1- Université de Rennes 1, Université Bretagne Loire, Service d'Anatomie et Cytologie Pathologiques, F-35042 Rennes, France
- 2- UMR 6290-IGDR, Rennes, France
- 3- Service d'Oncologie Médicale, Centre Eugène Marquis, Rennes, France
- 4- Ecole des Hautes Etudes en Santé Publique (EHESP), Rennes, France
- 5- Université de Rennes 1, Université Bretagne Loire, Service d'Urologie, F-35042 Rennes, France
- 6- Université de Rennes 1, Université Bretagne Loire, Service de Génétique Somatique des Cancers, F-35042 Rennes, France
- 7- Service d'Oncologie Médicale, CHU Saint-André, Bordeaux, France
- 8- Service d'Urologie, CHU Pellegrin, Bordeaux, France
- 9- Service d'Anatomie et Cytologie Pathologiques, CHU Pellegrin, Bordeaux, France
- 10- Université de Rennes 1, Université Bretagne Loire, Service de cytogénétique, F-35042 Rennes, France

Corresponding author:

Solene-Florence Kammerer-Jacquet

Service d'Anatomie Pathologique

CHU Pontchaillou

2 rue Henri Le Guilloux

35 033 Rennes cedex 9

Telephone number: 0033 2 99 28 42 79

Fax number: 0033 2 99 28 42 84

Email address: jacquet.sf@gmail.com

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MICROABSTRACT

Long-term responders (LTR) are defined by at least 18 months of response to sunitinib in metastatic clear cell renal cell carcinoma (ccRCC). Well described by clinical studies, the phenotype of these tumours has never been explored. Primary tumour of LTR demonstrated a different phenotype with PD-L1 low expression suggesting a potentially lower impact of targeted immunotherapy in these patients.

ABSTRACT

Background: Long-term responders (LTR) are defined by at least 18 months of response to sunitinib in metastatic clear cell renal cell carcinoma (ccRCC). Well described by clinical studies, the phenotype of these tumours has never been explored.

Patients and methods: In a retrospective and multicentre study, 90 ccRCC of metastatic patients were analysed. Immunohistochemistry (CAIX, VEGF, c-MET, PD-L1 and PD-1), *VHL* status were performed. Progression free survival (PFS) and overall survival (OS) were calculated from sunitinib introduction and from progression. LTR and their corresponding tumours were compared to others using univariate and multivariate analysis.

Results: Twenty-eight patients were LTR. They had a median PFS of 28 versus (vs) 4 months for other patients ($p<0.001$). Similarly, LTR had a median OS of 49 vs 14 months ($p<0.001$), even from progression (median 21 vs 7 months, $p=0.029$). They were associated with a favourable or intermediate risk (IMDC model) ($p=0.007$) and less liver metastasis ($p=0.036$). They experienced more frequent complete or partial responses at the first radiologic evaluation ($p=0.035$). The corresponding ccRCC were associated with less nucleolar ISUP grade 4 ($p=0.037$) and hilar fat infiltration ($p=0.006$). They were also associated with low PD-L1 expression ($p=0.02$). Only IMDC model and PD-L1 expression remained significant after multivariate analysis ($p=0.014$ and $p=0.029$, respectively).

Conclusion: Primary tumour characteristics of LTR were studied for the first time and demonstrated a different phenotype. Interestingly, they were characterized by low expression of PD-L1, suggesting a potentially lower impact of targeted immunotherapy in these patients.

Key words: renal cell carcinoma, sunitinib, PD-L1, long-term responders

INTRODUCTION

Clear cell renal cell carcinoma (ccRCC) is the most common histological subtype of renal cancer (70%).¹ Along with hereditary ccRCC, sporadic ccRCC is also frequently characterized by an alteration of the *VHL* gene, a tumour suppressor gene, leading to the transcription of genes regulated by HIF such as VEGF, which triggers the angiogenic process.² In 20% of patients, ccRCC is diagnosed at metastatic stage and 30% of the remaining patients will further develop metastases detected during follow-up. With an approximately 50% risk of metastasis, the prognosis of ccRCC is poor, and the mortality rate is 40% at 5 years.³

Anti-angiogenic therapies have significantly improved the prognosis of patients with metastatic clear cell renal cell carcinoma (ccRCC).⁴ Sunitinib, a tyrosine kinase inhibitor (TKI) targeting VEGFR, is currently an approved first-line treatment option for patients and is the most commonly administered treatment worldwide.^{5, 6} However, up to 30% patients experience progressive disease (PD) upon response evaluation criteria in solid tumours (RECIST) at their first evaluation and most patients ultimately develop PD.⁷ Although several resistance mechanisms have been described, few molecular markers of sensitivity or primary resistance with a prognostic impact have been identified.⁸

Another treatment approach is based on targeted immunotherapy using checkpoint inhibitors because ccRCC is considered an immunogenic tumour with high numbers of mononuclear immune cells such as tumour-infiltrating lymphocytes (TIL).⁹⁻¹¹ PD-L1 is a transmembrane protein which binds to its co-stimulatory receptor, PD-1 (B7-1), expressed by activated TILs, as a means to down-regulate antitumour immune responses by promoting TIL apoptosis and thus favour tumour progression. Recent clinical trial (Checkmate 025) demonstrated the superiority of anti-PD-1 (nivolumab) over everolimus in second-line treatment.¹² Following the results of Checkmate 214, the combination of nivolumab plus ipilimumab is now recommended as first-line therapy for patients with intermediate or poor prognosis according to International Metastatic Renal Cell Carcinoma Database Consortium (IMDC) model.^{13, 14}

Although numerous studies have focused on primary refractory patients defined by progression within the first 3 months, few have studied LTR.¹⁵ Molina *et al.* defined long-term response as a durable complete response or remaining progression-free for more than 18

months and clinically characterized them without assessing the phenotype of the tumours.¹⁶ With the advent of new treatment in ccRCC, it is crucial to better identify them as these patients are the most likely to benefit from sunitinib.¹³

In this study, we aimed to describe the pathological and immunohistochemical phenotype and *VHL* status of ccRCC and the clinical outcome of patients according to their long-term responder status.

MATERIAL AND METHODS

Patient selection and classification

Primary ccRCC-specimens were collected from patients undergoing nephrectomy in two French University Hospitals (Rennes and Bordeaux) from 1997 to 2013 with metastases either at diagnosis or during the follow up. For inclusion in the study, patients with metastatic ccRCC received sunitinib (50mg/day, four weeks-on/two weeks-off) as first-line treatment (prior cytokine therapy was allowed) and completed at least one 28-day cycle of sunitinib, and undergone their first CTscan assessment with Response Evaluation Criteria In Solid Tumours (RECIST 1.1).¹⁷ Drug schedule and dose-reduction policy complied with local practice guidelines. Follow-up chest/abdomen CT-scans were performed every 2 cycles of treatment (3 months). LTR were defined by at least 18 months of treatment without any progression.¹⁶ For each patient, the following clinical and pathologic information was gathered (using data base UroCCR): age, sex, the six factors in the IMDC model (anaemia, thrombocytosis, neutrophilia, Karnofsky performance status <80, <1 year from diagnosis to first-line targeted therapy and hypercalcemia) before sunitinib introduction, pTNM stage at nephrectomy, tumour size, and nucleolar ISUP grade.^{18, 19} Histopathologic assessment was performed by three experienced pathologists (SFKJ, MY and NRL). For each patient, frozen ccRCC were available. Informed consent was signed from each patient and institutional review board approval was obtained for this study (CNIL declaration receipt 1812601v0).

Immunohistochemical study

For each primary tumour, a representative slide of the tumour with the highest nucleolar grade and the corresponding paraffin block was selected. Four µm-thick whole tissue sections were cut and mounted on glass slides (Superfrost+, Menzel Glazer). The preparations were dried for 1 hour at 58°C, and then overnight at 37°C. The sections were deparaffinized with toluene

and rehydrated with ethanol. The preparations were pretreated and immunostained using Ventana Benchmark XT. VEGFA (Anti-VEGF antibody, sc-152, dilution 1/100 ; Santa Cruz Biotechnology, Santa Cruz, CA, USA), CAIX (Anti-CAIX antibody, ab15086, dilution 1/1500, Abcam, Cambridge, UK), c-MET (Anti-Total c-MET, SP44, Rabbit Monoclonal Primary Antibody, ready-diluted, Ventana, Roche, Switzerland), PD-L1 (Anti-PD-L1 antibody, clone 130021, dilution 1/200, RD System, Minneapolis, USA) and PD1 (anti-PD-1 antibody, clone NAT105, dilution 1:50; Abcam, Cambridge, UK) expressions were assessed by immunohistochemistry as previously described.²⁰⁻²² The reactivity of antibodies was revealed with HRP-labeled polymer conjugated secondary antibodies using diaminobenzidine (DAB) as chromogen (Sigma-Aldrich, France). Negative controls were performed by omitting the primary antibody. The tumour expression for each antibody was independently evaluated (SFKJ and NRL), without knowledge of the case. The cut-off for positive cases was 30% of tumour cells for VEGF and 85% for CAIX as previously described.^{20, 21} For PD-L1 and MET, absent (0), weak (1), moderate (2) and strong expression (3) were reported and cases were then subdivided into negative (0–1) or positive (2–3) subgroups.^{22, 23} For PD-1, immunostaining density was evaluated in tumour infiltrating lymphocytes and was semi-quantified as absent, rare, moderate or dense as previously reported.²⁴

VHL status

Next generation sequencing

For *VHL* gene, the entire coding sequence and exon-intron junctions of exons 1, 2 and 3 were analyzed. Genomic DNA was extracted using Magstration System 12GC (Bionobis) according to the manufacturer's instruction. Genomic DNA from all samples was quantitated with the Quan-iT PicoGreen dsDNA assay kit (Thermo Fisher Scientific). DNA target preparation and enrichment were performed by amplification using the Access Array® system (Fluidigm, San Francisco, USA). A 10-nucleotide “barcode” tag, specific to each sample and Illumina-specific sequencing adaptors were attached using secondary PCR. Purified products were then pooled and sequenced on the Illumina MiSeq NGS instrument (Illumina Inc., San Diego, California).

Multiplex Ligation-dependent Probe Amplification

Methylation-Specific-MLPA (MS-MLPA) was used to detect CpG methylation islands in *VHL* gene promotor. The SALSA MS-MLPA kit ME001B Tumour suppressor-1 allows detecting aberrant methylation of CpG-Islands located in the promoter region of the *VHL* gene.²⁵ The unmethylated DNA will not generate a signal, and a normal probe signal will be

detected if the site is methylated.

Statistical analysis

The phenotype of ccRCC (histologic and immunohistochemical features, *VHL* status in long term responders) was compared with other patients. Associations were analyzed with χ^2 , Fisher and Mann-Whitney tests. For logistic regression, we used a backward stepwise selection with $p < 0.05$ inclusion criteria. The rank of elimination was obtained when a variable was removed from the equation, and the odds ratio, 95% CI, and p-value for the removed variables were obtained on the removal step. For clinical outcome, we represented progression-free survival (PFS) from the sunitinib introduction to progression and overall survival (OS) from sunitinib introduction to death using Kaplan-Meier curves. We also represented PFS starting from 18 months for LTR and OS from progression to death. All p-values were 2-sided, and p-values less than 0.05 were considered statistically significant. All statistical analyses were performed using Stata 14.1 (College Station, TX) software.

RESULTS

Patient and tumour characteristics

Patient and tumour characteristics are presented in Table 1. The study retrospectively included 90 consecutive metastatic patients with primary ccRCC. The mean follow-up period was 25 months (range 1-76 months) from sunitinib introduction. The median PFS and OS were 10 and 22 months, respectively. Eighty-five patients (94.4%) experienced progression and 71 (78.9%) died from their cancer. Men were most represented (62.2% versus 37.8%). According to the IMDC model, most patients were in the intermediate group (47.7%). Locally advanced tumours were mostly represented (stage pT3-T4 in 75.5% of patients), and showed a high nucleolar ISUP grade 3 or 4 ($n=88$, 92.3%). Metastases were present at the initial diagnosis for 55.6% of patients. More than two-third of patients presented multiple metastatic sites ($n=69$; 76.7%). The most common metastatic sites were lung (75.6%), bone (53.3%) and liver (23.3%).

VHL status

VHL status was assessed in the entire cohort ($n=90$). All patients were negative for germ-line mutations. A *VHL* gene mutation was observed in 64 cases (71.1%). Mutations occurred in exons 1, 2 and 3 in 28 (43.8%), 21 (32.8%) and 15 cases (23.4%) respectively. Stop,

frameshift, missense, and splice site mutations were detected in 8 (12.5%), 34 (53.1%), 18 (28.1%) and 4 (6.3%) cases respectively. *VHL* promoter methylation occurred in 10 cases (11.1%). At least one or more *VHL* abnormalities (*VHL* inactivation) were observed in 74 cases (82.2%).

Correlation with clinical outcome

Twenty-eight patients were LTR and belonged to good (n=10), intermediate (n=15) and poor (n=3) risks according to IMDC score. Among LTR, 23 patients progressed and 13 received second-line therapy (everolimus, n=5; pazopanib, n=3; sorafenib, n=3; axitinib, n=2), 7 received third-line therapy (everolimus, n=5; sorafenib, n=1 and axitinib, n=1) and 1 received a fourth-line therapy by pazopanib. Twelve patients were last known to be alive with a continuing response or stable disease. Kaplan Meier curves for PFS and OS are presented in Figures 1, 2 and 3. LTR had a median PFS of 28 months versus 4 months for other patients ($p<0.001$). Similarly, LTR had a median OS of 49 months versus 14 months for other patients ($p<0.001$). From progression, LTR still had a difference of survival, median OS of 21 versus 7 months ($p=0.29$).

Pathological, immunohistochemical phenotype and *VHL* status of LTR

LTR had good or intermediate prognosis according to the IMDC model ($p=0.007$) and less liver metastasis ($p=0.036$) (Table 2). They more frequently experienced a complete or partial response at the first radiologic evaluation ($p=0.035$). Corresponding ccRCC were associated with less nucleolar ISUP grade 4 ($p=0.037$) and less hilar fat infiltration ($p=0.006$). They were also associated with low expression of PD-L1 by immunohistochemistry ($p=0.02$), Figure 4. No association with *VHL* status was identified. All the significant variables were included for logistic regression except liver metastases that could interfere with Heng score criteria. The only 2 factors that remained significantly associated with LTR were good or intermediate risk (IMDC model) ($p=0.014$, OR (95% CI)=5.29 (1.39, 20.01)) and low PD-L1 expression ($p=0.029$, OR (95% CI)=3.145 (1.22, 8.31)), Table 3.

DISCUSSION

To our knowledge, this is the first study to assess the phenotype of ccRCC in LTR along with a long term clinical follow-up. Previous studies identified clinic-biological criteria associated with LTR.

Molina *et al.* described for the first time the patients who benefit the most from sunitinib at clinical level ¹⁶. This retrospective study (n=186) mainly included ccRCC but also other subtypes representing 12% of their cohort even though their carcinogenesis was different. Moreover, the majority of them (52.1%) received sunitinib in combination with gefitinib, bevacizumab or everolimus whose targets were not the same. In their study, favourable Memorial Sloan Kettering Cancer Center (MSKCC) score was associated with LTR.

A recent study by Escudier *et al.* proposed a pooled analysis of clinical trials including retrospectively 5714 patients.²⁶ Among them, 897 (15.7%) patients were LTR. They were associated with higher percentage of early tumour shrinkage at the first scan, white race, favourable criteria of IMDC mode, clear cell histology, no liver metastasis, body mass index ≥ 25 kg/m².

Similarly, in our study, LTR exhibited good or intermediate prognosis (IMDC model related to MSKCC), which remained unsurprisingly significant after multivariate analysis. Moreover, they had significantly fewer liver metastases than other patients. Indeed, liver metastases were previously associated with poor prognosis in patients with metastatic ccRCC and the absence of liver metastases was already correlated to LTR.^{26, 27} McKay *et al* previously described that their impact was attributed to alteration of sunitinib metabolism and the liver microenvironment that could favour an aggressive phenotype.²⁷

One limitation of our study is the selection of 18 months as a cut-off to define LTR but was reproducible with previous studies.^{16, 26} Another limitation is our sample size impairing multivariate analysis. However, contrary to previous studies, we included only clear cell histology and performed a pathological and immunohistochemical study with *VHL* status on primary tumours along with clinical data.

At molecular level, *VHL* was inactivated by mutually exclusive mutation or promoter methylation in the majority of cases without any association with the sunitinib response. As previously described, no association between *VHL* status and outcome was observed.^{28, 29} *VHL* inactivation, considered as an archetypical tumour-initiating event in ccRCC carcinogenesis, failed to identify sunitinib responders, as other mechanisms probably interfere.

We report a distinct pathological and immunohistochemical phenotype of the primary tumours of LTR. As a matter of fact, ccRCC were particularly associated with a lower nucleolar ISUP grade and a less frequent infiltration of hilar fat. They were independently

associated with lower expression of PD-L1 by immunohistochemistry. First demonstrated in our study, this is consistent with the results of Choueiri *et al.* who correlated PD-L1 expression with poor outcome in patients with metastatic ccRCC.³⁰

Recent update on metastatic ccRCC treatment based on recent Checkmate 214 results recommends a dichotomized approach for first line therapy according to IMDC model.¹³ In intermediate- and poor risk patients, targeted immunotherapies are now indicated whereas in favourable-risk patients, antiangiogenic therapy remains the standard of treatment. In our study, some LTR belonged to intermediate- and poor risk and could have been treated by immunotherapies according to the new standards; however, they clearly benefited from sunitinib. The high expression of PD-L1 associated with better response to immunotherapy in Checkmate 214 trial, could suggest, in LTR associated with low expression of PD-L1, less interest in targeted immunotherapies and reinforce its potential use as predictive biomarker.

Interestingly, the OS was also found to be prolonged in these patients, even from progression. LTR demonstrated an increased overall survival that was not only explained by their response to sunitinib first-line treatment, but also to second-line targeted therapy. The hypothesis could be that these patients who are more than good responders to sunitinib may be likely to do well under other targeted therapy. Further studies could help clarify whether such responses are specific to sunitinib or reflect underlying favourable biology.

In conclusion, LTR showed a prolonged OS even from progression. Their primary tumours demonstrated a different phenotype with PD-L1 low expression suggesting a potentially lower impact of targeted immunotherapy in these patients.

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AUTHORS' CONTRIBUTION

SF Kammerer-Jacquet, N Rioux-Leclercq: Protocol/project development

A Brunot, B Peyronnet, G Verhoest, R Mathieu, B Laguerre, A Ravaud, JC Bernhard, F Dupuis, M Yacoub: Data collection or management
 M Lefort, S Bayat, A Lespagnol, J Mosser: Data analysis
 SF Kammerer-Jacquet, MA Belaud-Rotureau, N Rioux-Leclercq, K Bensalah: Manuscript writing/editing

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

RESEARCH INVOLVING HUMAN PARTICIPANTS AND/OR ANIMALS

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. For this type of study formal consent is not required.

INFORMED CONSENT

Informed consent was obtained from all individual participants included in the study.

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ACCEPTED MANUSCRIPT

Tables

Table 1: Patient and tumour characteristics of 90 metastatic ccRCC .

Characteristics	No	%
Age (years)		
Mean		61,3
Range		37-85
Sexe		
Male	56	62,2%
Female	34	37,8%
IMDC		
Favourable	20	22,2%
Intermediate	43	47,8%
Poor	27	30,0%
T stage		
T1	14	15,6%
T2	8	8,9%
T3	63	70,0%
T4	5	5,6%
N stage		
N0	75	83,3%
N1-N2	15	16,7%
M stage		
M0	40	44,4%
M1	50	55,6%
Tumor size (cm)		
Mean		9.6
Range		2-9.5
ISUP nucleolar grade		
Grade 2	7	7,8%
Grade 3	33	36,7%
Grade 4	50	55,6%
RECIST 1		
Complete response	1	1,1%
Partial response	27	30,0%
Stable disease	34	37,8%
Progression disease	28	31,1%

Table 2: Comparison between long-term responders and other patients.

Variables	Long-term responders (n=28)	%	Other patients (n=62)	%	p-value
Clinical and radiological data					
Male	20	71,4%	36	58,1%	0,226 †
Age >65	8	28,6%	29	46,8%	0,104 †
Good/intermediate risk (IMDC)	25	89,3%	38	61,3%	0,007 †
Distant lymph node metastasis	11	39,3%	28	45,2%	0,603 †
Pulmonary metastasis	20	71,4%	48	77,4%	0,54 †
Bone metastasis	16	57,1%	32	51,6%	0,626 †
Liver metastasis	2	7,1%	19	30,6%	0,015 †
Cerebral metastasis	4	14,3%	15	24,2%	0,286 †
Multiple metastasis	21	75,0%	48	77,4%	0,802 †
RECIST 1 (CR-PR vs SD-PD)	13	46,4%	15	24,2%	0,035 †
Pathological analysis					
Size >7cm	16	57,1%	42	67,7%	0,331 †
Nucleolar ISUP grade 4	11	39,3%	39	62,9%	0,037 †
Sarcomatoid component	3	10,7%	16	25,8%	0,104 †
Tumor necrosis	19	67,9%	52	83,9%	0,085 †
Microvascular invasion	11	39,3%	30	48,4%	0,422 †
T3-T4 stage	19	67,9%	49	79,0%	0,253 †
Hilar fat infiltration	7	25,0%	35	56,5%	0,006 †
Peri-renal fat infiltration	14	50,0%	31	50,0%	1 †
Venal invasion	14	50,0%	27	43,5%	0,569 †
N1-N2 stage	3	10,7%	12	19,4%	0,375 ‡
M1 stage	15	53,6%	35	56,5%	0,799 †
Immunohistochemistry					
CAIX >85%	10	35,7%	18	29,0%	0,526 †
VEGFA >30%	12	42,9%	38	61,3%	0,103 †
MET 2-3 intensity	18	64,3%	44	71,0%	0,526 †
PD-L1 2-3 intensity	16	57,1%	50	80,6%	0,02 †
PD-1 moderate or dense	18	64,3%	48	77,4%	0,192 †
VHL status					
VHL mutation	20	71,4%	44	71,0%	0,964 †
Promoter methylation	2	7,1%	8	12,9%	0,718 ‡
VHL inactivation	22	78,6%	52	83,9%	0,543 †

†, χ^2 test; ‡, Fisher exact test

RECIST 1: First RECIST evaluation, CR, complete response; PR, partial response; SD, stable disease; PD, progression disease

Table 3: Logistic regression with rank of elimination and intermediate results

Variables	Rank	OR	95% CI	p-value
Good/intermediate prognosis (Heng)	-	5.292	1.399, 20.01	0.014
RECIST 1 (CR-PR vs SD-PD)	3	0.389	0.139, 1.083	0.071
Nucleolar ISUP grade 4	1	0.509	0.182, 1.419	0.197
Hilar fat infiltration	2	0.401	0.136, 1.184	0.098
PD-L1 low intensity	-	3.145	1.122, 8.812	0.029

Supplementary table

Variables	Long-term responders (n=28)		Short-term responders (n=62)		p-value
		%		%	
Heng score					
Favourable	10	35,7%	10	16,1%	0.039 †
Intermediate	15	53,6%	28	45,2%	0.46 †
Poor	3	10,7%	24	38,7%	0,007 †

Figure 1: Kaplan Meier curve representing progression-free survival (PFS) and overall survival (OS)

PFS: Median survival: 4 vs 28 months ($p < 0.001$), OS: median survival: 14 vs 49 months ($p < 0.001$)

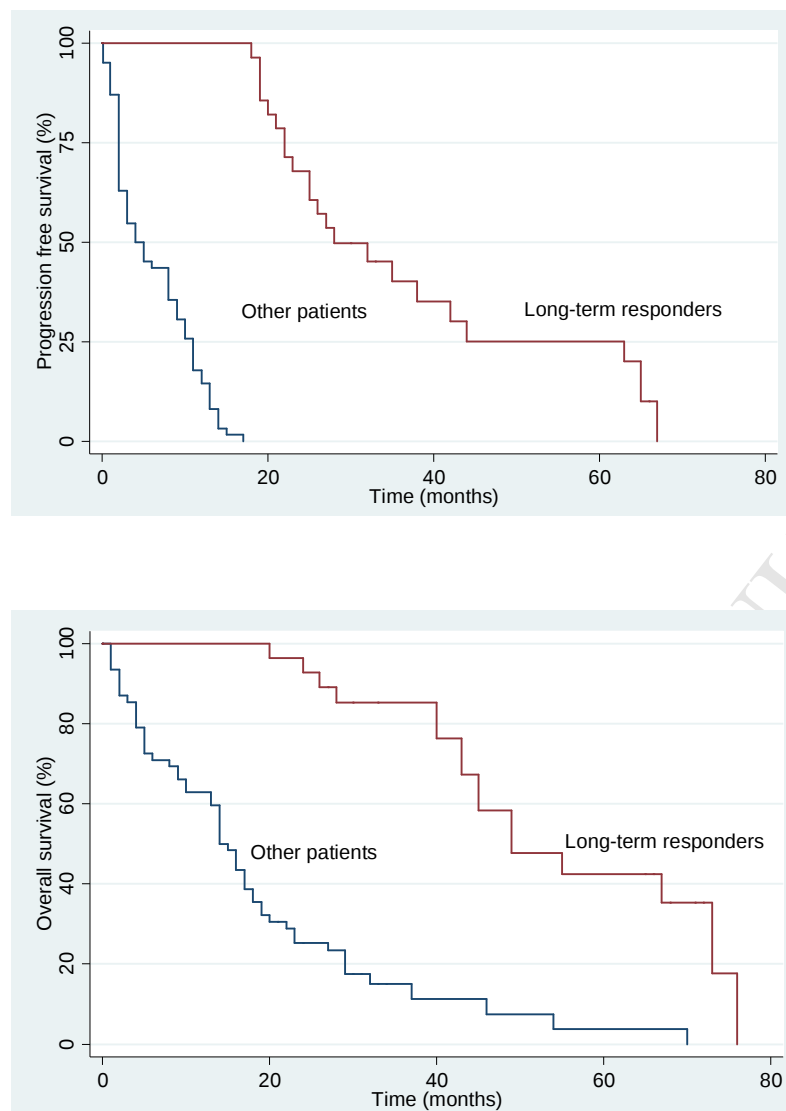


Figure 2: Kaplan Meier curve representing progression free survival of long-term responders (n=28) with time zero starting at 18 months.

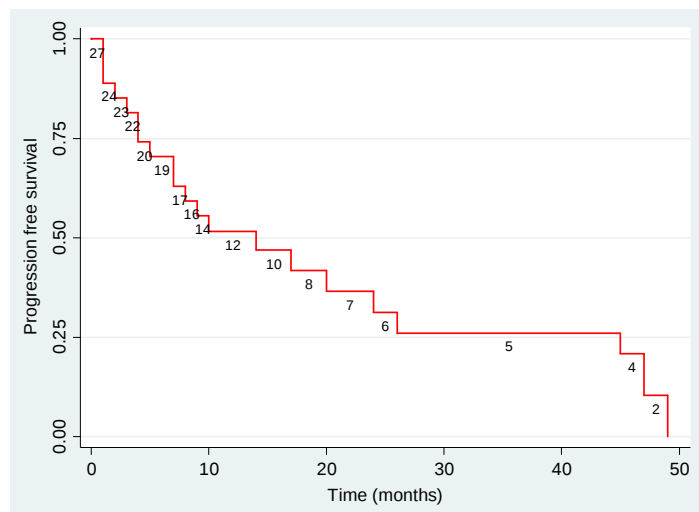


Figure 3: Kaplan Meier curves representing overall survival (OS) from progression.

OS: median survival: 7 vs 21 months ($p=0.029$)

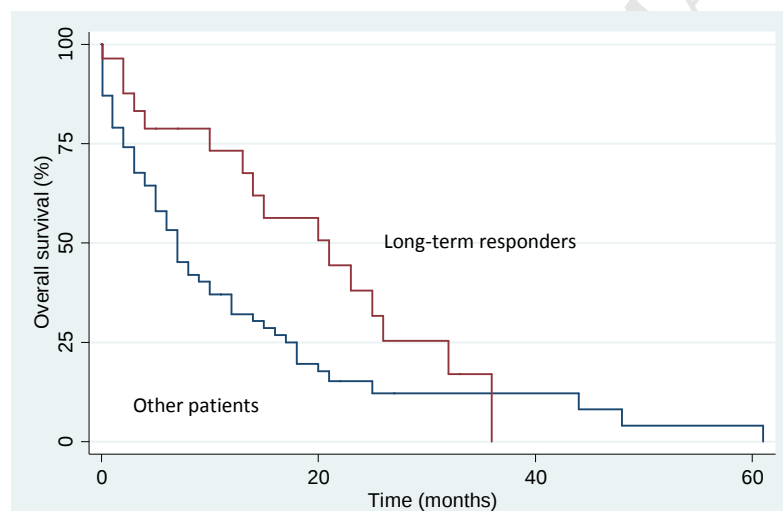


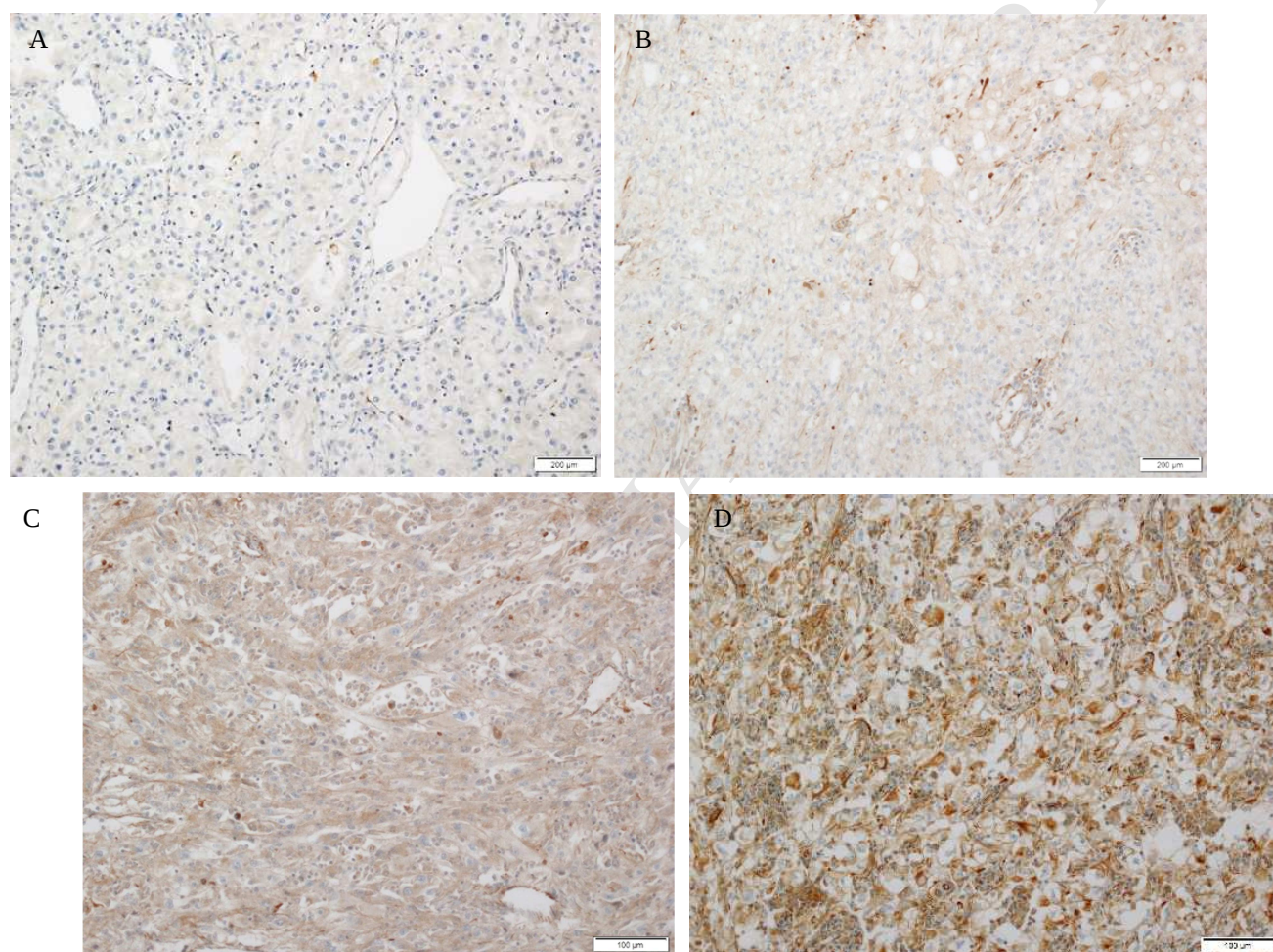
Figure 4: PD-L1 expression

A- Absence of PDL1 expression, immunohistochemistry (IHC) x100

B- Low expression of PDL1, IHC x100

C- Moderate expression of PDL1, IHC x100

D- High expression of PDL1, IHC x100



Supplementary figure: Kaplan Meier curve representing progression-free survival (PFS) and overall survival (OS) according to PD-L1 intensity

PFS: Median survival: 8 vs 17 months ($p=0.27$), OS: Median survival: 18 vs 29 months ($p=0.30$)

